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Direct activation of a mouse *Hoxd11* axial expression enhancer by Gdf11/Smad signalling

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ABSTRACT

A *Hoxd11/lacZ* reporter, expressed with a *Hoxd11*-like axial expression pattern in transgenic mouse embryos, is stimulated in tailbud fragments when cultured in presence of Gdf11, a TGF-β growth/differentiation factor. The same construct is also stimulated by Gdf11 when transiently transfected into cultures of HepG2 cells. Stimulation of the reporter in HepG2 cells is enhanced where it contains only the 332 bp *Hoxd11* enhancer region VIII upstream or downstream of a *luciferase* or *lacZ* reporter. This enhancer contains three elements conserved from fish to mice, one of which has the sequence of a Smad3/4 binding element. Mutation of this motif inhibits the ability of Gdf11 to enhance reporter activity in the HepG2 cell assay. Chromatin immunoprecipitation experiments show direct evidence of Smad2/3 protein binding to the *Hoxd11* region VIII enhancer. The action of Gdf11 upon *Hoxd11* in HepG2 cells is inhibited, at least in part, by SIS3, a specific inhibitor of Smad3. SIS3 also produces partial inhibition of *Hoxd11/lacZ* expression in cultured transgenic tailbuds, indicating that Smad3 may play a similar role in the embryonic expression of *Hoxd11*. Transgenic mouse experiments show that the Smad binding motif is essential for the axial expression of *Hoxd11/lacZ* reporter in the embryo tailbud, posterior mesoderm and neurectoderm.

Introduction

The body plan of an animal is first laid down in its embryo as a pre-plan of developmental gene expression. This provides patterns, in space and time, of transcription factors and signalling molecules which mediate the specification of cell fate. Patterns of developmental gene expression are commonly regulated by *cis*-regulatory elements, which include enhancers and silencers of gene expression, and which are located outside the coding sequence. Mutational changes in *cis*-regulatory elements of developmental genes have frequently mediated evolutionary changes in body morphologies (Gaunt and Paul, 2012; Wittkopp and Kalay, 2012).

The *Hoxd11* expression boundary plays a role in specifying position of the lumbosacral junction along the vertebral column (Davis and Capecchi, 1994). *Hoxd11* function overlaps with that of *Hoxa11* and *Hoxc11*, and loss of all six alleles is required for complete absence of sacral vertebrae (Wellik and Capecchi, 2003). A 332 bp *Hoxd11* enhancer fragment (region VIII) (Gerard et al., 1993; Gerard et al., 1997; Zakany et al., 1997) is located downstream of the coding region, between *Hoxd11* and *Hoxd10*. Region VIII also serves as an enhancer for *Hoxd10*. Thus, embryos homozygously deficient for region VIII show posterior shift of *Hoxd11* and *Hoxd10* expressions at 9 days gestation, and a subsequent posterior shift of the lumbosacral junction (Zakany et al., 1997). Surprisingly, expression boundaries of these two Hox genes become normal by 10 days gestation.

The lumbosacral and thoracolumbar junctions are shifted posteriorly in *Gdf11* knockout mice. This is due to posterior shifts within paraxial mesoderm in the anterior expression limits of Hox genes that pattern these regions (McPherron et al., 1999). These mice, with dramatically elongated torsos, have been called dachshund mice (Gad and Tam, 1999). It is unclear whether Gdf11 signalling acts directly or indirectly upon the Hox genes. In a converse experimental protocol, Gdf11 overexpression in chick embryo neural tissue results in forward shift in the anterior expression limits of posteriorly-expressed Hox genes (Liu, 2006). It appears that Gdf11 defines Hox gene expression domains in the spinal cord in co-operation with FGF (Liu, 2006; Liu et al., 2001). Together, the above studies indicate that Gdf11 is able to regulate Hox expression boundary positions in both paraxial mesoderm and neural tissue of the posterior embryo. The hindlimb position of *Gdf11* mutant mice is also shifted caudally (McPherron et al., 1999). This position is set as part of the trunk-tail

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transition, co-ordinated by *Gdf11* through an effect upon *Isl1*, rather than upon Hox genes (Jurberg et al., 2013). *Gdf11* is expressed in the mouse embryo tailbud from about 8 to beyond 12.5 days (Gamer et al., 1999; McPherron et al., 1999; Nakashima et al., 1999). The *Hoxd11* expression boundary becomes established in the tailbud commencing at about 8.75 days (Izpisua-Belmonte et al., 1991).

Gdf11 is a member of the TGF-β family of growth factors (Feng and Derynck, 2005; Massague et al., 2005; Shi and Massague, 2003; Wu and Hill, 2009). These bind to complexes of type I and type II serine/threonine kinase receptors at the cell surface. Predominantly, Gdf11's effect upon axial patterning utilizes the type I receptor ALK5 (Andersson et al., 2006), and the type II receptor Activin IIB (AcvrIIB), supplemented by AcvrIIA (Oh et al., 2002). Knockout of either *ALK5* or *AcvrIIB* results in axial defects similar to *Gdf11* knockout, with similar posterior shifts in a variety of posteriorly-expressed Hox genes (Andersson et al., 2006; Oh and Li, 1997; Oh et al., 2002).

Binding of TGF-ß ligand to the type II receptor recruits and phosphorylates the type I receptor which then, in turn, phosphorylates a cytoplasmic receptor-regulated Smad protein (R-Smad). Alk5 phosphorylates the closely related Smad2 and Smad3 proteins (Andersson et al., 2006). Evidence has been presented that it is Smad2 that mediates Gdf11 activation of Hox gene expression (Liu, 2006). Once activated, R-Smad complexes with Smad4, a Co-Smad, to promote both nuclear accumulation and binding to specific enhancer motifs (Smad binding elements or SBEs) for direct regulation of gene expression. The Smad3 and Smad4 DNA binding element contains a repeated AGAC sequence or its reverse complement, GTCT (Dennler et al., 1998), with the palindrome GTCTAGAC as the optimal binding sequence (Zawel et al., 1998). Smad2 itself normally lacks DNA binding activity but may bind to this motif via its complex with Smad4 (Feng and Derynck, 2005).

We now show that Gdf11 exerts a stimulatory effect upon a *Hoxd11/lacZ* transgene expressed in the mouse embryo tailbud. *Hoxd11* reporters are also activated by Gdf11 in HepG2 cells, and our studies indicate that this is a direct effect upon the region VIII enhancer, operating via Smad signalling to a Smad binding element. Transgenic mouse experiments show that the Smad binding motif is also essential for the expression of *Hoxd11/lacZ* reporter in the embryo tailbud.

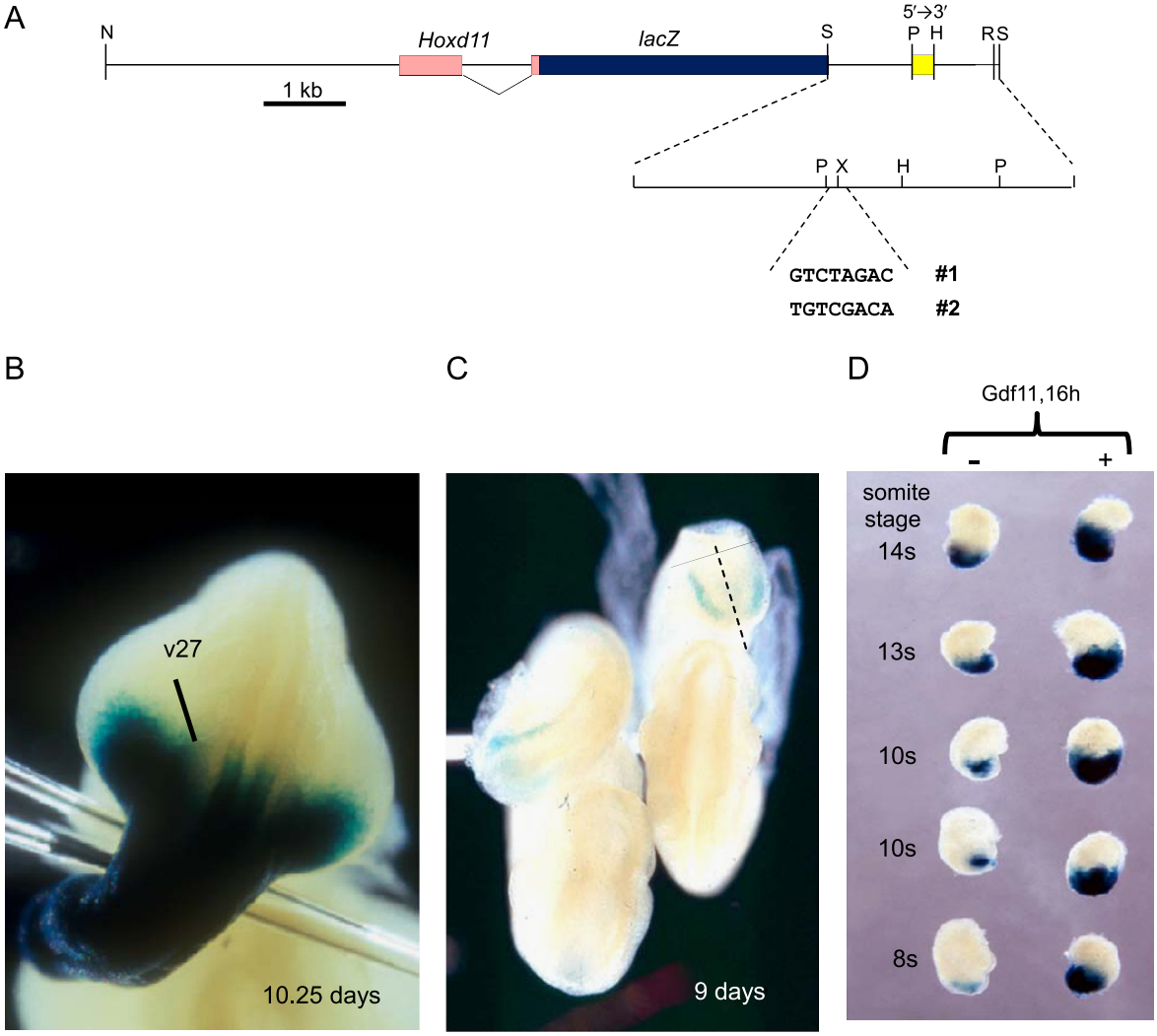

Fig. 1. *Hoxd11/lacZ* transgene expression in embryos is stimulated by Gdf11. (A) *Hoxd11/lacZ* reporter constructs #1 and #2. In #2 a putative Smad binding motif (GTCTAGAC) is mutated. Red box, *Hoxd11* coding sequence; yellow box, *Pst*1/*Hin*dIII 332 bp fragment which contains the region VIII enhancer; N, *Nsi*1; S, *Spe*1; P, *Pst*1; H, *Hin*dIII; R, *Eco*R1; X, *Xba*1. (B) A transgenic embryo line expresses construct #1 with an anterior axial boundary at the level of prevertebra 27 (v27), shown here at 10.25 days, and similar to expression of the endogenous *Hoxd11* gene. (C) Transgene expression commences in the tailbud at 9 days. (D) Tailbuds from 8.75 day transgenic embryos were bisected along the lines shown in C. Left-hand fragments were cultured without, and right-hand fragments with, Gdf11 at 500 ng/ml for 16 h prior to staining for lacZ activity. Fragments are viewed with anterior at the top. Stages of the embryos (8-somite to 14-somite) at the onset of culture are indicated. s, somite.

Materials and methods

DNA constructs

The long *Hoxd11/lacZ* construct (construct #1) (Fig. 1A) contains 7.3 kb of mouse *Hoxd11* DNA. It is similar to the Ns-E (*Nsi*1-*Eco*R1) construct characterized earlier (Gerard et al., 1993), but differs as follows: (i) *lacZ/SV40* polyA is targeted in frame by recombineering (Liu et al., 2003) into the second exon of *Hoxd11* after nucleotide 90 of the homeobox; (ii) the 3′ end of the *Hoxd11* second exon is omitted, together with the first 58 base pairs downstream of the *Hoxd11* stop codon; and (iii) a *Spe*1 site is introduced at the 3′ end of *lacZ*. The latter allows replacement of *Hoxd11* downstream sequences with a corresponding 2.16 kb *Spe*1 fragment mutated within the putative Smad binding motif of the *Hoxd11* region VIII (construct #2) (Fig. 1A). The mutation converts an *Xba*1 site into a diagnostic *Sal*1 site. A short *Hoxd11/lacZ* construct (construct #3) comprises the 332 bp *Pst1*/*Hin*dIII fragment that includes region VIII, inverted relative to its normal orientation and followed in series by *SV40* minimal promoter, Kozak motif (GCCGCCACC), full *lacZ* coding sequence and *SV40* polyA (Fig. 2A). This construct is comparable with the P-H/hs construct characterized earlier (Gerard et al., 1993). All *lacZ* constructs were prepared in *Bluescript KS*$^-$ (Stratagene). Construct #4, made in *pGL3-promoter* (Promega), was similar to construct #3 but with *luciferase* as the reporter gene.

Various mutations were introduced by PCR into the conserved motifs of region VIII as shown in Fig. 4. Region y (construct #6), z (construct #7) and x (construct #8) mutations introduce diagnostic *Sal*1, *Eco*R1 or *Spe*1 sites. Further constructs were prepared in which the *Hoxd11* region VIII enhancer fragment is located in both orientations upstream (constructs #4 and #9) and downstream (constructs #10 and #11) of *luciferase* in the *pGL3-promoter* vector (Fig. 5A).

Hoxc11 299 bp fragment homologous to *Hoxd11* region VIII was isolated by PCR using primers CACCTTCATACTCCTTGGATG (5′) and ATGTGGGGCAAAGGCATGATG (3′), and was then subcloned into *pGL3-promoter* (Promega).

Expression constructs were made by cloning full-length coding sequences of *Cdx1*, *Hoxd11* or *Hoxd10*, with Kozak motif upstream of ATG into a *pCAGGS* vector. Expression, driven by the constitutive *CAGGS* promoter, was confirmed in HepG2 cells by immunofluorescence (not shown).

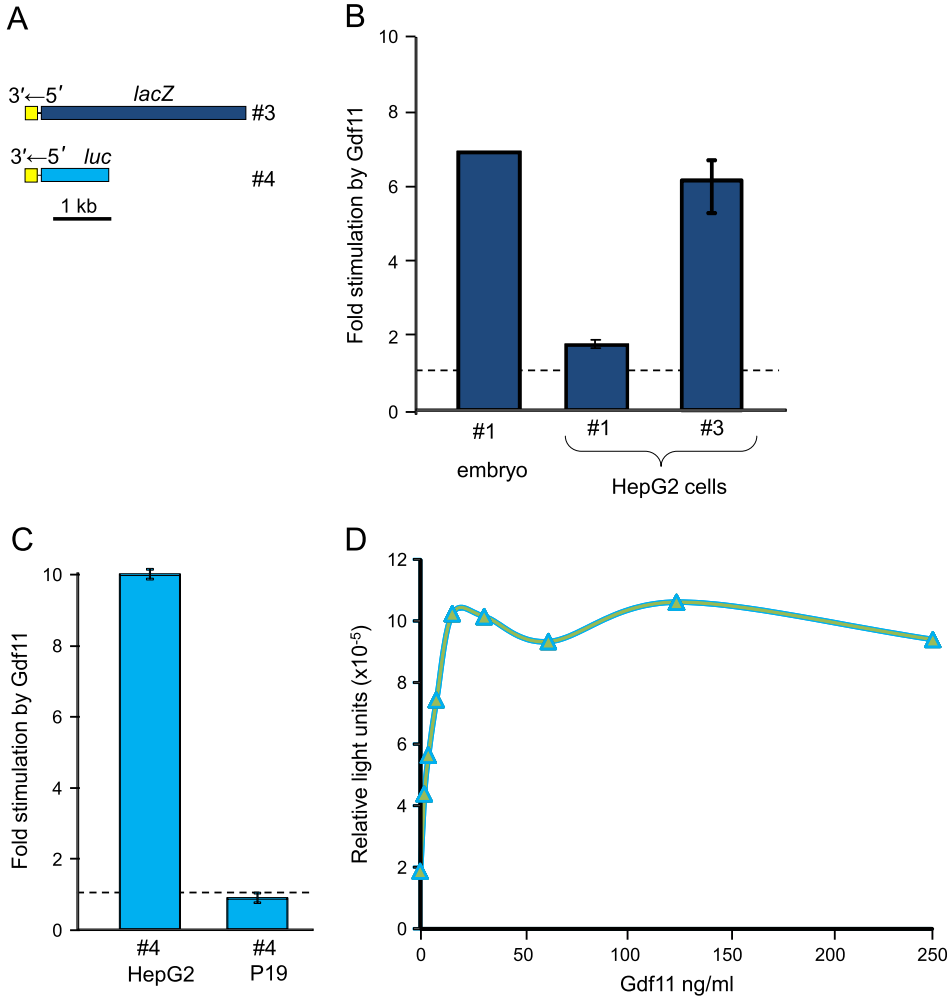

Fig. 2. Chemiluminescence to detect Gdf11 stimulation of *Hoxd11* reporters. (A) *Hoxd11* region VIII enhancer (yellow box) upstream of minimal *SV40* promoter and either *lacZ* (construct #3) or *luciferase (luc)* (construct #4) reporter genes. (B) Gdf11 stimulation in transgenic embryo tailbud or HepG2 cell monolayers. (C) Gdf11 stimulates construct #4 in HepG2 but not P19 cells. In B and C, fold stimulation is the effect of 16 h (embryo) or 21 h (cell monolayer) incubation in Gdf11 (500 ng/ml for embryos; 50 ng/ml for cells) relative to no Gdf11. The embryo data compares activity in one half of a bisected embryo tailbud cultured with Gdf11, relative to the other half not given Gdf11 (latter shown as dotted baseline) (cf. Fig. 1D). The cell culture data within each plot are from a single experiment, and each graph bar shows average values for three replicate cultures given Gdf11, relative to the average for three replicates not given Gdf11 (latter shown as dotted baseline). Range bars are shown. (D) Dose-response curve for Gdf11 stimulation of construct #4 in HepG2 cells.

Reporter expression analysis in embryos and HepG2 cells

Transgenic mouse lines or transient transgenic embryos were prepared by injecting the insert from the long *Hoxd11/lacZ* constructs #1 or #2 (Fig. 1A) into pronuclear mouse eggs, as described earlier (Gaunt et al., 2003). Tailbuds from 8.75 day *Hoxd11/lacZ* embryos were bisected using tungsten needles along the lines shown in Fig. 1C, and then cultured in 50% D-MEM; 50% heat-inactivated rat serum in 5% CO_2; 95% air, at 37 °C for 16 h. Post-culture embryo fragments were either stained for lacZ activity (Hogan, 1994) or lysed for β-galactosidase chemiluminescence assay (Applied Biosystems). Human Gdf11 and Wnt3a were from R&D Systems; human FGF2 was from Invitrogen; *all-trans* retinoic acid was from Sigma; and SIS3 inhibitor was from Calbiochem.

HepG2 cells, from ATCC, were cultured on gelatinized surfaces in Dulbecco's MEM with glutamine and 10% foetal bovine serum. Transfections and assays for β-galactosidase and luciferase were all as described earlier (Gaunt and Paul, 2011). A stably-transgenic cell line, HepG2/d11/lacZ, was prepared from HepG2 by transfection of a linearized variant of construct #3 incorporating a neomycin resistance cassette, followed by selection in 1.6 mg/ml G418. After one week, and detachment of dead cells, G418 was reduced to 666 μg/ml for colony growth and subsequent maintenance of a transgenic line.

Chromatin immunoprecipitation (ChIP) analysis

We used the Smad2/3 ExactaChIP kit (R&D Systems), which includes an antibody that recognizes conserved epitopes in Smad2 and Smad3 proteins (Brown et al., 2007). HepG2/d11/lacZ transgenic cells at near-confluence in a 90 mm dish were treated for 18 h with 50 ng/ml Gdf11. Medium was then replaced with PBS at 37 °C containing 1% formaldehyde. After 9 min, this was changed to PBS containing 0.125 M glycine. After a further 5 min, cells were scraped into PBS, pelleted, and then taken up in 300 μl of the ExactaChIP lysis buffer containing protease inhibitors (Sigma P8340). Chromatin was reduced to 200–1000 bp fragment length by sonication in a Diagenode Bioruptor. Immunoprecipitation and reverse crosslinking were as described in the ExactaChIP instruction manual, using streptavidin agarose beads (Sigma 85881) that had been pre-blocked in 2% BSA for 1 h at 4 °C. PCR primers for mouse *Hoxd11* Region VIII were: CTGCAGTGCCCTTTATGTCACAGGAC (5′) and AAGCTTTGGCAATTTAAAAAATTC (3′), and for human *alpha1 actin* were: GGCGTCATGGTCGGTATGGGT (5′) and CGCACGATCTCGCGCTCAG (3′). Forty three cycles of PCR were used.

Results

Hoxd11/lacZ transgene expression in embryos is stimulated by Gdf11

Embryos of 10.25 days gestation from a mouse line transgenic for the *Hoxd11/lacZ* construct #1 (Fig. 1A) show an anterior boundary of expression at the level of presumptive prevertebra 27 (Fig. 1B). This is the same as that published for a similar *Hoxd11/lacZ* transgene (Gerard et al., 1993), and similar to that of the endogenous *Hoxd11* gene (Izpisua-Belmonte et al., 1991). Hoxd11/lacZ activity is first seen at 9 days (Fig. 1C). Left- and right-hand halves from the tailbuds of 8.75 day *Hoxd11/lacZ* embryos, prepared by cuts along the lines shown in Fig. 1C, were cultured for 16 h in absence or presence of Gdf11, followed by lacZ staining. The stimulatory effect of Gdf11 was clear by increased intensity and spatial extent of lacZ staining (Fig. 1D). The enhanced staining does not extend up to the anteriormost parts of the fragments, suggesting that tissue becomes refractory to Gdf11 at a certain distance from the posterior end. In chemiluminescence assays for β-galactosidase activity we find that Gdf11 protein stimulates, by up to 7-fold, the expression of lacZ in bisected tailbuds of *Hoxd11/lacZ* transgenic embryos (Fig. 2B).

Gdf11 stimulates Hoxd11 reporters in HepG2 cells

HepG2 cells are one of only a few cell culture types known to respond to Gdf11 (Andersson et al., 2006). When these human cells are transfected with the mouse *Hoxd11/lacZ* reporter construct #1, we observe 1.5 fold stimulation by Gdf11 protein (Fig. 2B). However, this increases to more than 5 fold (Fig. 2B) when the 332 bp enhancer fragment region VIII (Gerard et al., 1993) is used upstream of a minimal *SV40* promoter and *lacZ* (construct #3; Fig. 2A). This shortened construct, with enhancer reversed relative to its endogenous orientation, is similar to one known to be expressed with an approximately normal anterior boundary in transgenic mouse embryos (Gerard et al., 1993). Construct #4 is similar to construct #3 but uses luciferase as the reporter. This is also stimulated in HepG2 cells, though not in P19 mouse embryonal carcinoma cells (Fig. 2C). Fig. 2D shows a dose response curve for the action of Gdf11 upon expression of construct #4 in HepG2 cells. Maximal effect is obtained at a dose equal to, or greater than, 15 ng/ml.

Conserved regions in the Hoxd11 enhancer

When we examine the sequence of the 332 bp *Hoxd11*enhancer fragment region VIII we find three adjacent regions (*x*, *y* and *z*) conserved from fish to mouse (Fig. 3A) (Gerard et al., 1997). Region y contains the palindrome GTCTAGAC. This is remarkable because this sequence has been identified as an optimal binding motif for Smad3 and Smad4 proteins (Zawel et al., 1998), two components in the TGF-β signalling pathway. Region z has the structure of a conserved C/A/TATAAA motif, a recognition motif for Cdx proteins (Margalit et al., 1993) which are known to affect Hox gene expression. We find conserved regions y and z at a corresponding position downstream of *Hoxc11* (Fig. 3B), though not of *Hoxa11* (not shown). Interestingly, the Hoxc and d loci are thought to have arisen by split of a common ancestral locus (Ravi et al., 2009). *Hoxc11*, unlike *Hoxd11*, does not have conserved region x adjacent to region y (Fig. 3).

Mutation of conserved regions affects response to Gdf11

In Fig. 4A and B we examine the effect of Gdf11 upon expression of luciferase construct #4 in HepG2 cells, and show the effects of mutations within the conserved motifs x, y and z of the *Hoxd11* enhancer. Wild-type enhancer (construct #4) is stimulated more than 5 fold by Gdf11. In contrast, Gdf11 stimulation is reduced when the *Hoxd11* enhancer is absent (construct #5) or is present with either region y (construct #6) or region x (construct #8) mutated. Mutation within region z has no apparent effect upon Gdf11 stimulation (construct #7).

Since conserved region *z* has the sequence of a Cdx protein binding motif we tested construct #4 for a possible synergistic effect of co-transfection with a Cdx1 expression construct. This did not produce an obvious effect upon the level of Gdf11 stimulation (Supplementary Fig. S2A). We also tested co-transfections with expression constructs for two other homeobox genes: *Hoxd10* and *Hoxd11*. Here, we were testing for possible autoregulatory effects, synergistic with Gdf11, of Hoxd10 and Hoxd11 proteins upon their own enhancer. Neither Hoxd10 nor Hoxd11 produce a substantial effect upon the level of Gdf11 stimulation (Supplementary Fig. S2A).

We also tested for possible synergistic effects of retinoic acid, Wnt3a, and FGF, three signalling molecules known to be present in the embryo tailbud. No substantial effects were seen upon Gdf11

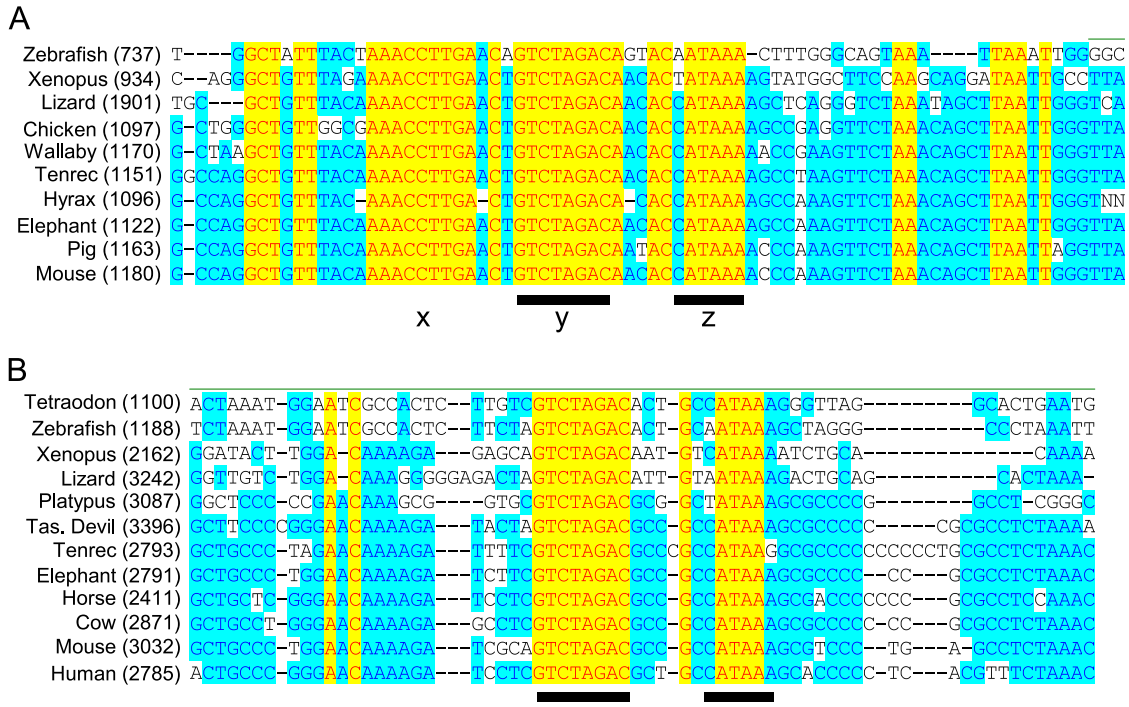

Fig. 3. Evolutionarily conserved motifs within *Hoxd11* region VIII and *Hoxc11*. (A) *Hoxd11*. (B) *Hoxc11*. Numbers indicate base pairs downstream of the *Hox11* gene stop codon. x, y and z are regions of conserved sequence. Bars underline putative binding sites for Smad (region y) and Cdx (z) proteins. Sequences aligned using Vector NTI (Invitrogen). Yellow highlight, fully conserved bases; blue, highly conserved. Accession numbers of sequences analyzed are given in Supplementary Fig. S1. Lizard, Anole Lizard; Tas. Devil, Tasmanian Devil.

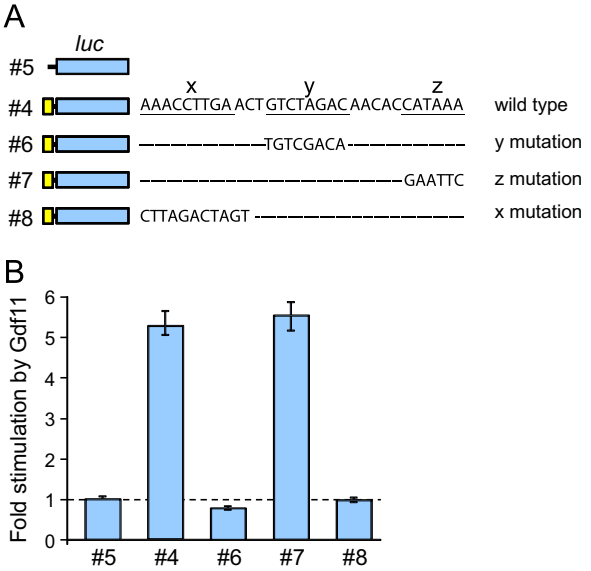

Fig. 4. Effects of mutations upon Gdf11 activation of mouse *Hoxd11* region VIII in HepG2 cells. (A) construct #4 (Fig. 2) and its variants which either lack *Hoxd11* enhancer (yellow box) or contain within it mutations introduced to evolutionarily conserved regions x, y and z. Dashes indicate identity with the wild-type mouse construct #4. (B) Effect of mutations upon Gdf11 activation of *Hoxd11* region VIII in HepG2 cells. In B, fold stimulation shows the effect of 21 h incubation in Gdf11 (50 ng/ml), relative to no Gdf11. Graph, range bars and baselines as in Fig. 2.

stimulation of construct #4 in HepG2 cells (Supplementary Fig. S2B). This is in spite of the fact that HepG2 cells are known to possess receptors for retinoic acid (Zhang et al., 2010), Wnt3a (Ceballos et al., 2011), and FGF (Hu et al., 2012). The small but reproducible suppression seen for FGF was also obtained at higher doses (10, 50, and 100 ng/ml.) (not shown).

In these experiments we have examined transcription and signalling factors for possible synergistic effects upon Gdf11 activation of the *Hoxd11* region VIII enhancer. Synergistic effects are described, for example, for the *Cdx1* enhancer (Prinos et al., 2001).The transcription and signalling factors tested alone, in absence of Gdf11, also failed to activate construct #4 (not shown), but our experiments do not rule out the possibilities that they may act upon *Hoxd11* outside the region VIII enhancer, or that there may be other factors not tested here that act inside or outside the enhancer.

Orientation of the Hoxd11 enhancer

In Fig. 5A and B, we examine the effect of the orientation of the 332 bp *Hoxd11*enhancer fragment in *luciferase*/minimal *SV40* promoter constructs. All orientations provide stimulation. However, greatest stimulation is obtained where the enhancer, either upstream (construct #4) or downstream (construct #11) of luciferase, is in reverse orientation relative to its endogenous sense. The full significance of this remains unclear since our reporter assays use circular plasmids. Interestingly, however, the *Hoxd11*enhancer upstream of *lacZ* had to be in reverse orientation in order to produce a near-normal pattern of lacZ expression in *lacZ* reporter transgenic embryos (Gerard et al., 1993).

An equivalent set of four constructs made with the *Hoxc11* homologous region showed little evidence of activation by Gdf11 (Fig. 5B). This is perhaps not surprising since the 299 bp *Hoxc11* fragment tested does not have the conserved region x (Fig. 3), which is essential for Gdf11 activation of the *Hoxd11* enhancer (Fig. 4A and B).

Gdf11 stimulation is reduced by SIS3

SIS3, a specific inhibitor of Smad3, mediates its effect by dose-dependent suppression of Smad3 phosphorylation, Smad3-DNA binding, and interaction of Smad3 with Smad4. SIS3 does not

affect phosphorylation of Smad2, or the expression of Smad4 (Jinnin et al., 2006). In HepG2 cells, Gdf11 activation of *Hoxd11/luciferase* reporter construct #4 is progressively inhibited by increasing doses of SIS3 (Fig. 6A). This suggests that it is Smad3 that, at least in part, mediates the effect of Gdf11 upon *Hoxd11* in HepG2 cells.

To test for a role of Smad3 in the embryo, we bisected 8.75 day *Hoxd11/lacZ* transgenic embryo tailbuds and then compared left and right-hand fragments after culture for 16 h in either SIS3 inhibitor or DMSO control. Fragments cultured in SIS3 were reduced in lacZ expression as determined by lacZ staining, though inhibition was incomplete, even at 20 μM (Fig. 6B). This suggests that at least part of the Gdf11 effect upon embryonic *Hoxd11* expression may be mediated by Smad3.

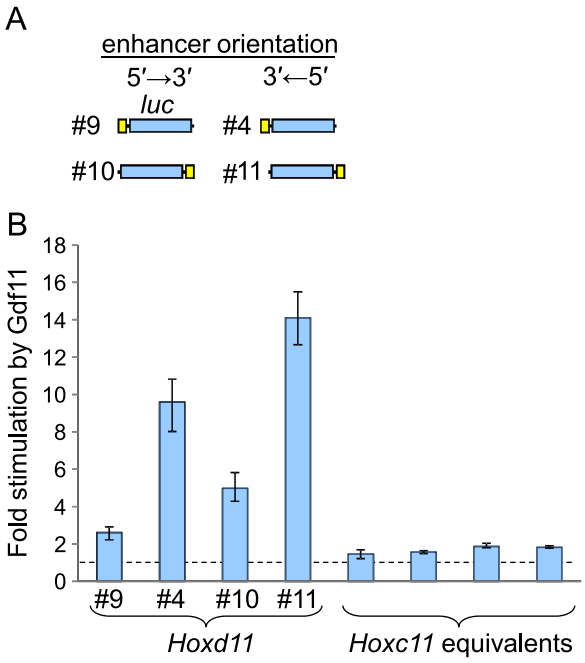

Fig. 5. Effects of position and orientation of the *Hoxd11* region VIII enhancer. (A) *Hoxd11* region VIII enhancer (yellow box) in various positions relative to *SV40* minimal promoter/*luciferase*. (B) Effects of enhancer orientation and position upon Gdf11 stimulation of the *Hoxd11* constructs, relative to each other and also to equivalent *Hoxc11* constructs. Culture conditions, graphs, range bars and baseline as in Fig. 2.

Chromatin immunoprecipitation (ChIP) shows binding of Smad2/3 protein to the Hoxd11 region VIII enhancer

To test directly for Smad protein binding to the *Hoxd11* region VIII enhancer we carried out ChIP experiments using an antibody which binds to conserved epitopes in Smad2 and Smad3 proteins. HepG2 cells exposed to Gdf11 after transient transfection at near confluence of *Hoxd11/lacZ* reporter construct #3 showed fewer than 10% transfected cells (Fig. 7A). For the ChIP analysis, we therefore also used a HepG2/d11/lacZ transgenic line which, upon exposure to Gdf11, expresses lacZ in 100% of the cells (Fig. 7B). This line expresses lower levels of lacZ in absence of Gdf11 (Fig. 7C), likely due to endogenous Smad activity upon the *Hoxd11* region VIII transgene. After Gdf11 exposure, HepG2/d11/lacZ cell chromatin contained, prior to immunoprecipitation, readily detected mouse *Hoxd11* region VIII and human *alpha1 actin* DNAs, but Smad2/3 antibody precipitated only the former, indicating that Smad2/3 protein had been bound in chromatin to *Hoxd11* region VIII (Fig. 7D). After use of a similar number of PCR cycles (forty three) we did not obtain positive ChIP results from transiently transfected HepG2 cells (not shown), presumably due to the low transfection efficiency (Fig. 7A).

A Hoxd11 transgene with mutation in the Smad binding motif is not expressed in the embryo tailbud

We wished to test whether the Smad binding motif in region VIII enhancer is essential for the expression of *Hoxd11/lacZ* construct #1 transgene in the embryo tailbud, and hence in the posterior mesoderm and neurectoderm (Fig. 8A–E). For this, we prepared transgenic embryos expressing construct #2 (Fig. 8F–J). Construct #2 differs from construct #1 only by mutation in the

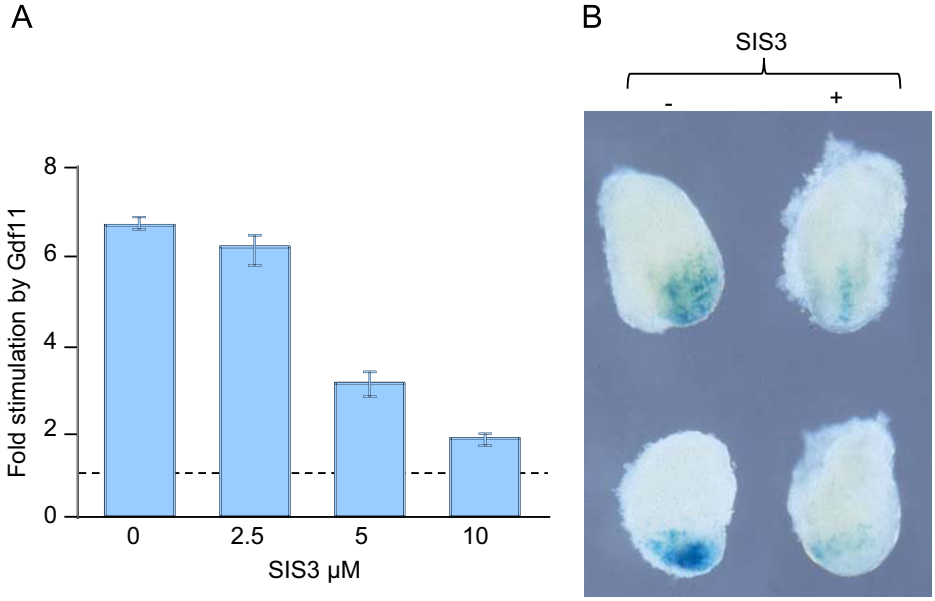

Fig. 6. Effect of specific inhibitor of Smad3 (SIS3) upon *Hoxd11* reporter expressions in HepG2 cells and embryo fragments. (A) Gdf11 stimulation of construct #4 is reduced by SIS3. The data are from a single experiment, and each graph bar shows average values for three replicate cultures given Gdf11, relative to the average for three replicates not given Gdf11 (latter shown as dotted baseline). Range bars are shown. All cultures received equal amounts of DMSO solvent. (B) Expression of Hoxd11/lacZ transgene in bisected 8.75 day embryo tailbud fragments, cultured without added Gdf11, is reduced by SIS3 (20 μM).

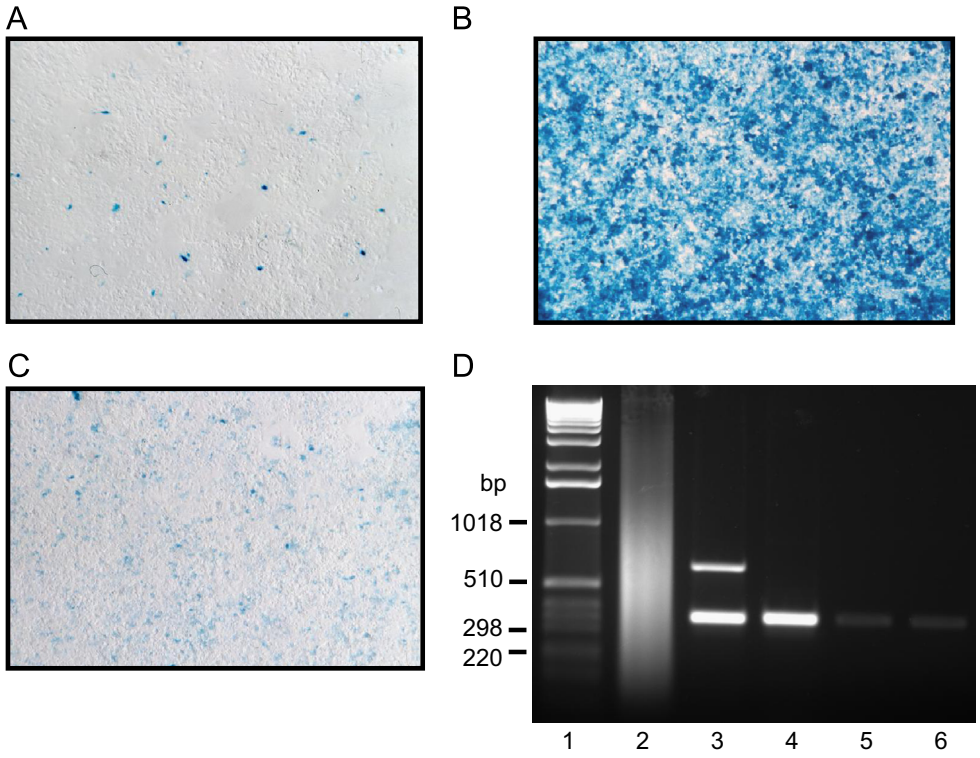

Fig. 7. Smad2/3 binding to the *Hoxd11* region VIII enhancer detected by chromatin immunoprecipitation (ChIP). (A) HepG2 cells transiently transfected with *Hoxd11/lacZ* construct #3, and treated for 18 h with Gdf11 (50 ng/ml) show fewer than 10% expressing cells (stained blue). (B) Cells stably transgenic for construct #3 (HepG2/d11/lacZ cells) show 100% lacZ positive cells after Gdf11 treatment, and lower levels of lacZ expression without Gdf11 (C). (D) ChIP analysis on transgenic HepG2/d11/lacZ cells treated with Gdf11. Lane 1, size markers (bp, base pairs); lane 2: chromatin prior to ChIP, sonicated to 200–1000 bp; lane 3: *Hoxd11* region VIII (332 bp) and *alpha1 actin* (713 bp) DNAs amplified by PCR from pre-ChIP chromatin; lane 4: *Hoxd11* but not *alpha1 actin* DNA amplified from chromatin precipitated by anti-Smad2/3 antibody; lanes 5,6: reduced *Hoxd11* amplification product after precipitation by normal goat IgG (lane 5) or no IgG (lane 6).

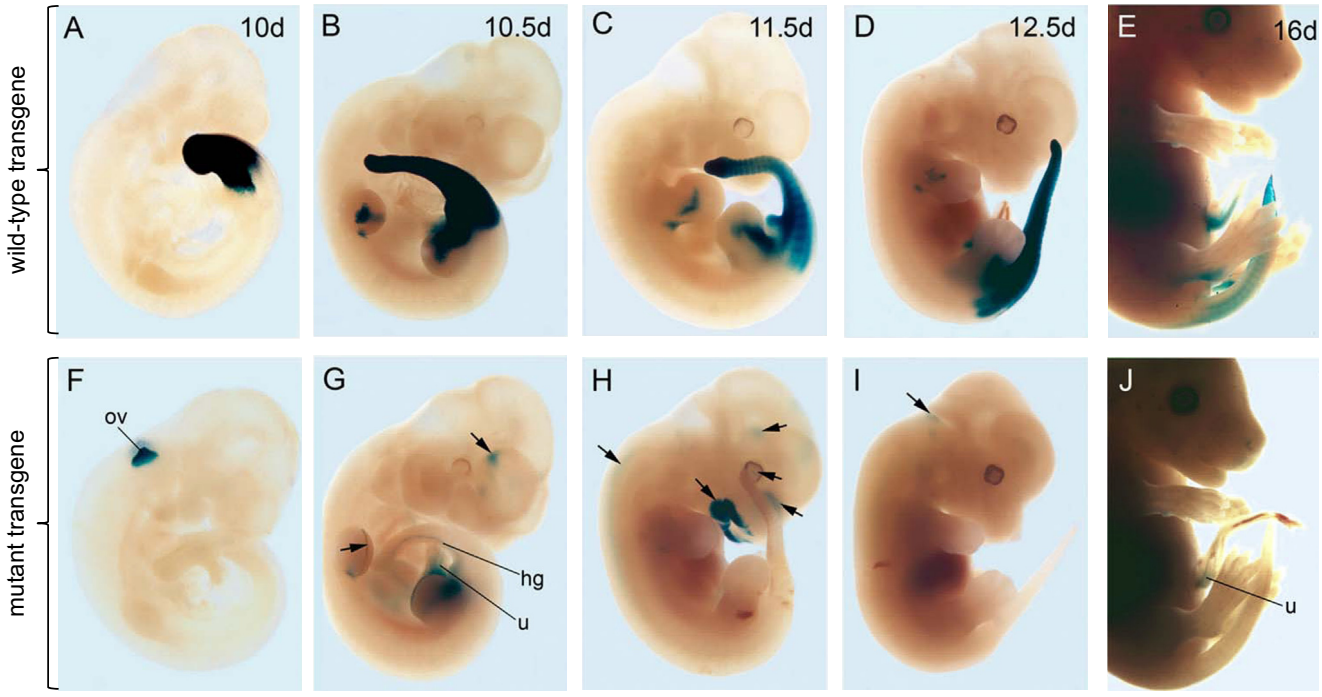

Fig. 8. Axial (tailbud) expression of *Hoxd11/lacZ* transgene in embryos is inhibited by mutation within the putative Smad binding motif. (A–E) Wild-type (construct #1) transgene expression in embryos at 10 to 16 days post coitum. (F–J) Mutant (construct #2) transgene expression in embryos at equivalent stages to those above. ov, otic vesicle; hg, hind gut; u, umbilicus. Arrows in G–I indicate other sites of ectopic (non-*Hoxd11*-like) expression.

putative Smad binding motif (conserved region y) of the *Hoxd11* region VIII enhancer (Figs. 1A and 3). In Fig. 8G–I, we show three independently-derived transient transgenic embryos for construct #2. None of these show the axial expression of lacZ in the tailbud, posterior mesoderm and neurectoderm as seen for construct #1at equivalent stages of development (Fig. 8B–D). Instead, expression of

construct #2 is expressed variably and ectopically between embryos in the head, apical ectodermal ridge, anterior neural tube, eye, mouth, and is also expressed at some sites shared with construct #1(hindgut and umbilicus). To examine a more complete range of embryonic stages we also made a transgenic line of mice that expresses construct #2 (Fig. 8F and J). At no stage from 10d (Fig. 8F) to 16d (Fig. 8J) does this line show *Hoxd11*-like expression in the tailbud. We conclude that the Smad binding motif in region VIII enhancer is essential at all stages from 10 to 16 days for axial expression of construct #1 in the tailbud, posterior mesoderm and neurectoderm, and that this is likely mediated by Gdf11/Smad signalling. We cannot rule out the possibility that additional *Hoxd11* Smad response elements might be located outside the limits of constructs #1 and #2. Gerard et al. (1993) examined embryos transiently transgenic for *Hoxd11/lacZ* constructs that lacked both the region VIII enhancer and additional downstream sequence. They detected no tailbud expression at 10 days, or later, consistent with our findings.

Discussion

Cis enhancers regulate axial Hox gene expression boundaries

Hox expression boundaries along the embryonic axis are established, at least to an important extent, as a result of regulatory factors interacting with *cis* enhancers located in and around the Hox genes. Thus, a near-normal pattern of expression has often been obtained simply from a *Hox/lacZ* transgene randomly integrated into the genome, provided it contains the appropriate *cis* elements. A good example here is given by *Hoxd11/lacZ* transgenes that include the region VIII enhancer (Gerard et al., 1993) (Figs. 1B and C, 8A–E). Factors that regulate Hox gene *cis* enhancers, directly or indirectly, may be soluble, diffusible substances such as retinoic acid, FGF and Gdf11 (Bel-Vialar et al., 2002; Gad and Tam, 1999), or intracellular transcription factors such as Cdx proteins (Charite et al., 1998; Gaunt et al., 2004).

In theory, such factors could affect the position of a Hox expression boundary along the axis either by affecting the initial timing of gene activation (temporal collinearity model) (Izpisua-Belmonte et al., 1991), or by their distribution being uneven (possibly graded) along the embryo (morphogen model). Gdf11, a TGF-β ligand, could be a morphogen for specifying expression domains of posteriorly-expressed Hox genes (McPherron et al., 1999). The known vertebrate morphogens activin and nodal both operate via TGF-β signalling. A model has been presented in which an anteroposterior gradient of Gdf11, operating in conjunction with an FGF gradient, specifies the anterior limits of Hox expression in the developing spinal cord (Liu, 2006). The implication here is that Hox expression boundaries form at specific threshold concentrations of Gdf11. Various other roles of TGF-β ligands are known to depend critically upon the concentration to which responding cells are exposed (Guzman-Ayala et al., 2009).

The Gdf11/Hoxd11activation pathway in embryos and HepG2 cells

The position of the lumbosacral junction, regulated in part by the *Hoxd11* expression boundary (Davis and Capecchi, 1994; Gerard et al., 1997; Zakany et al., 1997), is shifted posteriorly in Gdf11 knockout mice (McPherron et al., 1999). Consistent with this we show that expression of a *Hoxd11/lacZ* transgene is enhanced, and its domain expanded anteriorly by addition of Gdf11 to cultured embryo tailbud fragments.

Using HepG2 cells, we present the first evidence that Gdf11/Smad signalling acts directly upon a Hox gene enhancer, namely region VIII of *Hoxd11* (Gerard et al., 1993). Chromatin immunoprecipitation experiments showed binding of Smad2/3 protein to the region VIII enhancer in HepG2 cells transgenic for a region VIII/lacZ transgene. Two conserved elements are shown to be essential for function: a motif with the sequence (GTCTAGAC) of a Smad binding element and an adjacent, upstream motif. The function of the upstream motif is unknown. One possibility is that it is a transcription factor binding site, and it is known that Smad binding elements commonly lie adjacent to motifs bound by cell-type specific transcription factors (Mullen et al., 2011). It is the binding of such a transcription factor that renders the nearby Smad motif functional (Mullen et al., 2011). Mullen et al. (2011) suggest that this is mediated by opening of chromatin structure, although the applicability of this in our studies with transiently transfected plasmids is unclear. Apart from *Hoxd11*, several other posteriorly-expressed Hox genes are responsive to disruption in Gdf11 signalling (Andersson et al., 2006; McPherron et al., 1999; Oh and Li, 1997). It therefore seems likely that our Gdf11 assays will be applicable to other Hox gene enhancers. A homologous region including a putative Smad binding motif downstream of *Hoxc11* did not respond to Gdf11, possibly due to absence of the adjacent upstream motif in the short (299 bp) fragment that we tested. *Hoxc11* expression has specifically been shown to be shifted posteriorly in embryos lacking Gdf11 (McPherron et al., 1999) or its AcvrIIB receptor (Oh and Li, 1997).

The Gdf11 pathway in the embryonic axis operates through Alk5 and, as such, is expected to activate both Smad2 and Smad3 (Andersson et al., 2006). Both of these Smads are expressed together in most of the tissues of the embryo (Dunn et al., 2004; Tremblay et al., 2000). Liu (2006) presented evidence that the effect of Gdf11 upon axial patterning can be mediated by Smad2. Thus, a constitutively activated form of Smad2 introduced by electroporation in chick embryos was able to induce *Hoxc9* and *Hoxc10* in spinal cord. Liu's finding does not, however, rule out a role for Smad3 and we found that SIS3, a specific inhibitor of Smad3 (Jinnin et al., 2006), is able both to inhibit the effect of Gdf11 upon *Hoxd11* reporter in HepG2 cells, and to partially suppress *Hoxd11/lacZ* transgene expression in cultured tailbud fragments. It is known that Smad2 and Smad3 operate together in other TGF-β mediated events in the embryo, for example activin and nodal regulated signalling in mesoderm formation and patterning (Dunn et al., 2004). Smad knockout mice do not resolve the relative importance of Smad2 and Smad3 in Hox patterning. *Smad2*+/− mice are phenotypically normal, while homozygous mutants die at 8.5 to 9.5 days (Waldrip et al., 1998). *Smad3*−/− mice are viable and not reported to have homeotic defects (Datto et al., 1999), but it remains possible that Smad2 and Smad3 regulate Hox genes with functional redundancy, as they are known to do at other loci (Dunn et al., 2004; Takimoto et al., 2010).

While a *Hoxd11/lacZ* reporter containing 7.3 kb of Hox DNA was expressed with a *Hoxd11*-like axial pattern in transgenic mice, this was inhibited by mutation within the putative Smad binding motif, GTCTAGAC, of the region VIII enhancer. This shows that all in vivo activation of the *Hoxd11* transgene operates through this motif, but it does not rule out the possibility that Smad binding elements more distant from *Hoxd11* might also regulate expression of the endogenous gene. Mouse embryos knocked out for region VIII show posterior shift in endogenous *Hoxd11* expression at 9 days, but by 10 days the boundary has assumed its normal position (Zakany et al., 1997). This suggests a second level of control upon the *Hoxd11* expression domain. This control might lie outside the limits of our *Hoxd11/lacZ* constructs since we did not see any axial expression of lacZ in 10 to 16 day transgenic embryos expressing the Smad motif-deficient construct.

Conclusions

Gdf11 exerts a stimulatory effect upon a *Hoxd11/lacZ* transgene expressed in the mouse embryo tailbud, and *Hoxd11* reporters are also activated by Gdf11 in cultured HepG2 cells. We show that

stimulation in HepG2 cells occurs via the *Hoxd11* region VIII enhancer. Both a Smad binding motif and an adjacent conserved element are essential for this function, and chromatin immunoprecipitation reveals Smad2/3 protein bound to the activated enhancer. Transgenic mouse experiments show that the Smad binding motif is also essential for axial expression of *Hoxd11/lacZ* reporter in the embryo. Our findings provide the first evidence that Hox genes may be directly regulated by Gdf11/Smad signalling in the embryo tailbud.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.08.025.

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